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1. Introduction

Antiestrogens, especially tamoxifen, have proven to be effective in the treatment of hormone-responsive breast cancer. In metastatic breast cancer, antiestrogens lead to a response in nearly one half of patients with estrogen receptor (ER)-positive primary tumor (1). Resistance to antiestrogens, however, is a serious clinical problem. About 40% of ER-positive tumors fail to respond to antiestrogen therapy, and most, if not all, breast tumor patients that initially respond to antiestrogens will eventually develop resistance (2). A better understanding of the mechanisms of antiestrogen resistance is therefore urgently needed.

A recent mutagenesis approach has identified three independent loci associated with antiestrogen resistance (3), and the target genes of two of the loci, BCAR1 and BCAR3, have been characterized. Interestingly, sequence analysis of BCAR1 demonstrated it to code for the docking protein p130Cas (Cas), which we and others have previously identified to be a key molecule in intracellular signaling pathways (4). Subsequent studies demonstrated that enhanced activation of Cas signaling can induce antiestrogen resistance, at least in cell culture conditions (5). Recent studies have demonstrated that Cas is likely to have a relevant role also in clinical breast cancer; studies on breast cancer samples have shown that high levels of Cas expression correlate with poor relapse-free and overall survival, and the response to tamoxifen therapy in patients with recurrent disease was found to be reduced in patients with primary tumors that expressed high levels of Cas (6).

Our **hypothesis** supported by our preliminary data was that Cas has an important causal role in the development of antiestrogen resistance. As a corollary, understanding of the pathways that Cas activates may identify key regulators of antiestrogen resistance and novel clinical targets for breast cancer treatment, and measurements of Cas signaling levels may provide useful prognostic information for breast cancer patients. In this grant, our **objective** was to test our hypothesis, and to identify and characterize the signaling pathways that mediate Cas-induced antiestrogen resistance.

2. Body of the Report

2. A. Specific Aims.

In order to meet the objectives outlined above, two specific aims were set forth in our grant application. **In the first aim**, our goal was to identify and characterize the signaling pathways that mediate Cas-dependent antiestrogen resistance in cultured cells. Our hypothesis was that the interaction of Cas with two signaling molecules, Crk and BCAR3, is required for Cas-dependent antiestrogen resistance. We further hypothesized that the Rac-JNK pathway forms a common pathway downstream of the Cas/Crk/BCAR3 signaling complex to mediate antiestrogen resistance; this working model was to be tested in Aim 1. Should this model prove to be *in*correct, our alternative plan was to utilize a novel function-based screening method to identify Cas-interacting proteins that mediate antiestrogen resistance. Depending on the nature of the interacting molecules, further experiments were to be planned to dissect their roles in antiestrogen resistance.

In the second aim, our goal was to identify the tyrosine residues in Cas that become phosphorylated in breast cancer cells. Cas activates signaling pathways by binding to Src homology 2 (SH2)-domain containing signaling molecules, such as Crk, in a tyrosine phosphorylation-dependent manner. Further, our preliminary studies indicated that hyperphosphorylation of Cas correlates with antiestrogen resistance. Thus, our objective was to employ two types of mass spectrometers in a multi-tiered strategy

to systematically map the tyrosine residues in Cas that become phosphorylated in breast cancer cells *in vivo*.

The following **tasks,** as outlined in the original **statement of work,** were set forth in order the accomplish the above specific aims:

Task 1 (addressing Aim 1).

Test our working model that the Rac-JNK pathway forms a common pathway downstream of the Cas/Crk/BCAR3 complex to mediate antiestrogen resistance (months 1-24). See diagram below.

a. Generate mammalian expression constructs of activated and dominant-negative forms of BCAR3; we already have constructs for Cas, Crk, Rac and the JNK pathway.

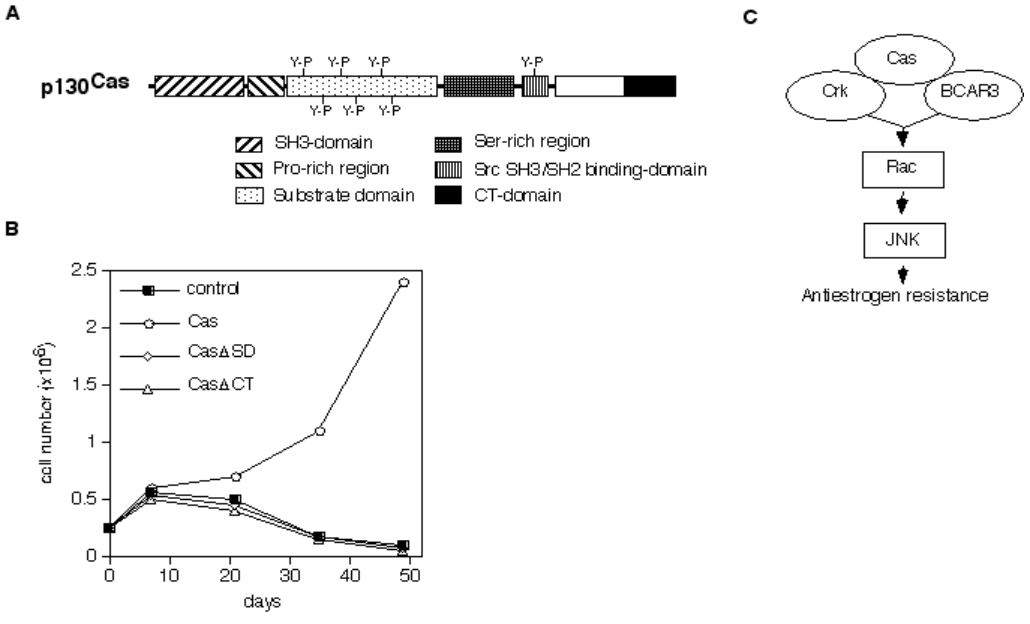
b. Test whether expression of an activated form of Crk and activated form of BCAR3 will rescue the CasDSD and CasDCT-phenotypes in antiestrogen resistance, respectively. In these studies, stable MCF-7 cell lines expressing the corresponding constructs will be generated, and cell proliferation in the presence of tamoxifen will be studied.

c. Test whether dominant-negative forms of Crk and BCAR3 block Cas-induced antiestrogen resistance. These studies will be performed as above.

d. Determine whether a dominant-negative form of Rac will block Cas-, Crk- and BCAR3-induced antiestrogen resistance. Also determine whether an activated form of Rac will rescue the defect in and inhibition of antiestrogen resistance by CasDSD and CasDCT, as well as by dominant-negative forms of Crk and BCAR3. These studies will be performed as above.

e. Determine what is the role of the JNK-pathway downstream of Rac in Cas-mediated antiestrogen resistance. The effect of overexpression of activated and dominant-negative JNK on Cas-,Crk-, BCAR3- and Rac-induced antiestrogen resistance will be studied.

Figure 1. A. Schematic presentation of the structure of Cas. **B.** MCF-7 cells transfected with the indicated plasmids were cultured in the presence of 1 μM of OH-tamoxifen. At indicated time points, three dishes of each transfectants were harvested, cells were counted, and reseeded at the initial density of 0.25 x 10^6 cells per 25 cm^2 flask. Cumulative cell numbers are presented over a 50-day culture period. **C.** Schematic presentation of the potential signaling pathways downstream of Cas that mediate antiestrogen resistance.



Outcome of studies outlined in Task 1:

We have completed the originally proposed studies outlined in Task 1, and are in progress with continuation studies beyond what was proposed in the DoD application for a (quality) manuscript completion.

As outlined in Task 1, our experimental strategy for testing our working model that the Rac-JNK pathway forms a common pathway downstream of the Cas/Crk/BCAR3 signaling complex to mediate antiestrogen resistance relied on reciprocal testing of dominant-negative and constitutively active forms of the various signaling molecules in this pathway. As such, bulk of our efforts initially focused on generating the genetic and cellular tools described above to be able to perform rigorous functional studies on the antiestrogen resistance in breast cancer cells. Accordingly, we generated the DNA constructs for activated and dominant-negative forms of BCAR3. Importantly, we also designed a point mutant construct of BCAR3 that specifically disrupts binding of BCAR3 to Cas, as opposed to other proteins utilizing the same protein-protein interaction domain within the BCAR3/SHEP proteins. Our recent collaborative paper with Dr. Pasquale's laboratory reported this finding (7). [Other constructs to be used in these studies have been described in our previous studies in refs (8-10)]. We apologize for not explicitly citing the DoD grant in this publication.

As a second step, we generated MCF-7 breast cancer cell lines expressing physiological or pathophysiological levels of the various constructs described in Task 1. To achieve this objective, we used DNA constructs generated in a retrovirus background, which allowed us to select either clones (to allow selection of a homogenous set of cells) or pools (to eliminate clonal variation) of MCF-7 cells expressing the desired levels of the exogenously delivered protein(s).

First, we selected MCF-7 cell lines overexpressing either Crk or BCAR3 in the CasΔSD or CasΔCT-expressing background, respectively. With these cell lines, we were able to assess the relative significance of Crk and BCAR3 signaling pathways in Cas-mediated antiestrogen resistance. That is, we were able to assess as to whether Crk and/or BCAR3 are *sufficient* components in antiestrogen resistance pathways downstream of certain Cas mutants. In short, the answer is no. Thus, overexpression of either Crk or BCAR3 had no impact in tamoxifen resistance in the cells.

Second, we generated cell lines expressing dominant-negative forms of either Crk or BCAR3 in the Cas-overexpressing MCF-7 background. These studies helped us to assess whether Crk and/or BCAR3 are *necessary* components in Cas-mediated antiestrogen resistance pathways. In short, the answer is yes. Thus, expression of a dominant-negative form of *either* Crk or BCAR3 abrogates the effect of Cas on antiestrogen resistance. In parallel, we generated breast cancer cell lines that overexpress wild-type and activated forms of Crk and BCAR3. Analysis of these cell lines confirmed the observation that Crk and BCAR3, by themselves, can induce antiestrogen resistance in breast cancer cell lines *in vitro*. Fourth, cell lines expressing either Cas, Crk, or BCAR3 were subjected to retroviral infection with a construct expressing a dominant-negative form of Rac. This line of investigation was expected to allow us to assess the putative *necessary* role of the small GTPase Rac in antiestrogen resistance mediated by Cas, Crk and/or BCAR3. Unfortunately, expression of dominant-negative form of Rac results in growth inhibition of the cells, thereby hindering our capability to directly assess the significance of this pathway in antiestrogen resistance. To overcome this technical problem, we generated breast cancer cell lines that express the activated form of Rac in a CasDSD or CasDCT-expressing MCF-7 background, and found that activated Rac indeed confers antiestrogen resistance in the cells. Thus, our original hypothesis appears to hold through. That is, both Crk and BCAR3 are necessary components

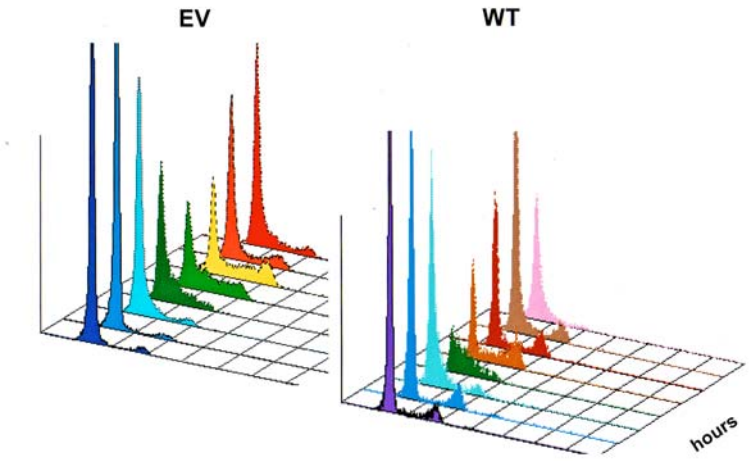
downstream of Cas to mediate antiestrogen resistance, and that the Cas/Crk/BCAR3 pathway converges on Rac for this function.

Current and future studies that build on the results obtained in Task 1.

Beyond Task 1, we will now assess how, exactly, the Cas/Crk/BCAR3/Rac mediates antiestrogen resistance, and the results obtained as a result of completing Task 1 have positioned us to be able to squarely address this. Thus, while the current status of our studies completes studies in Task 1, they fail to provide a significant breakthrough in the signaling field for a high-profile publication. Accordingly, the existence of the Cas/Crk/BCAR3/Rac has now been established in studies by us and others, but how this complex regulates such disparate biological activities as cell migration, cell spreading and antiestrogen resistance, remains completely unknown.

In order to leverage our completion of the Task 1 of this DoD grant into a high-profile publication, we have taken the following steps beyond the DoD grant. As noted above, we made the unexpected observation that dominant-negative form of Rac inhibits growth of (estrogen-dependent) breast cancer cells. While a technical hurdle for signaling studies, these results also provided the novel observation that the Cas/Crk/BCAR3/Rac could mediate cell proliferation, and that this previously unrecognized biological activity could lie at the heart of explaining how antiestrogen resistance might come about. To be able to tease out the putative role of this complex in cell cycle regulation, we have employed Cas -/- knock-out cells, and compared their growth profile to cells that have physiological levels of Cas. The advantage of this approach (as opposed to studies described above) is that we will not be expressing exogenous levels of Cas, but rather take the reverse approach and reduce Cas levels by genetic means. Importantly, we have uncovered, consistent with the possible role of cell cycle regulation linking the CAs/Crk/BCAR3/Rac complex to antiestrogen resistance, that Cas likely plays a role in cell cycle progression, and specifically affects the transition stage from G1 to S phase of cell cycle. This can be visualized in the FACS analysis of cells expressing wild-type Cas at physiological levels ("WT") or cells lacking Cas and instead expressing a control empty vector ("EV") (Fig. 2).

Figure 2: Absence of p130Cas leads to a delay in the cell cycle progression



Quantification of the FACS analysis is shown in Fig. 3, which demonstrates a delay of bulk of the cells lacking Cas in entering the S phase. While these cells eventually "catch up" (Fig. 4), we expect that enhanced Cas expression in breast cancer cells may lead to antiestrogen resistance by providing a growth advantage in the crucial G1-S transition of cell cycle. Studies are currently in the plans to address this in more detail. Importantly, previous studies have shown that the JNK pathway may provide feedback regulation to the Erk pathway, a known regulator of the G1-S transition via cyclin D1. Thus, this hypothesis is our current focus.

Figure 3:

Quantification of Cell Cycle Progression in EV and WT Cells

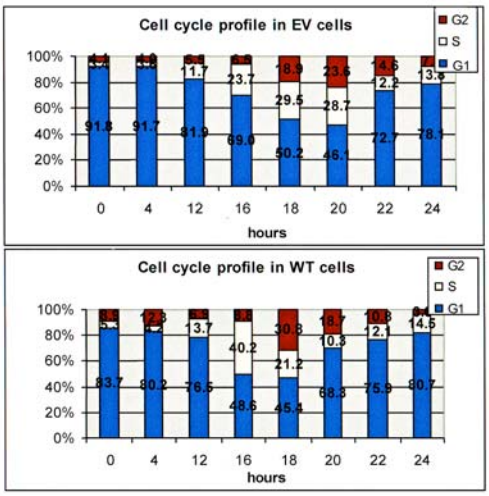
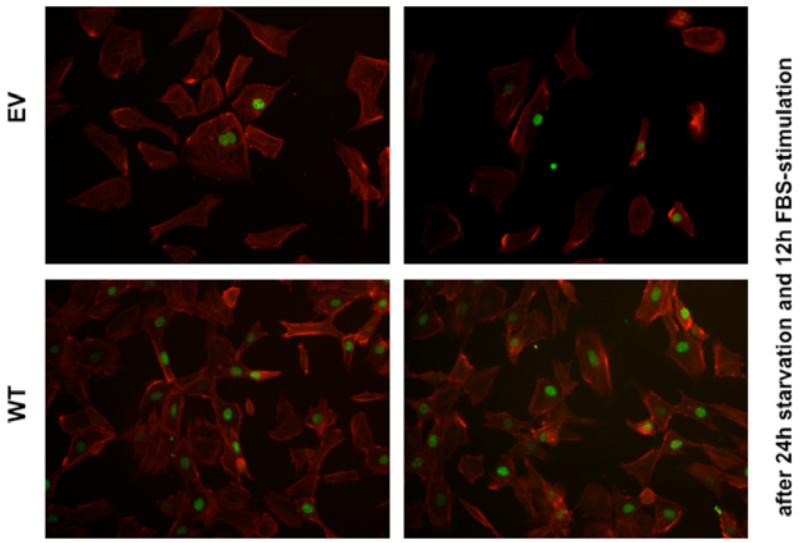


Figure 4:

Delay in Cell Cycle Progression correlated with different kinetics of BrdU Incorporation



Research that has benefited from studies outlined in Task 1:

Studies outlined above have addressed the biology of Cas in breast cancer. In parallel, DoD Predoctoral fellowship supported structural studies of Cas in breast cancer. While their was no financial overlap between the two projects, both studies benefited intellectually from each other. Thus, research results obtained in Task 1 significantly guided the following science (as listed by publications).

Nasertorabi, F., Garcia-Guzman, M., Briknarova, K., Larsen, E., Havert, M. L., Vuori, K. & Ely, K. R. (2004) Organization of functional domains in the docking protein p130Cas. **Biochem. Biophys. Res. Commun.** 324:993-998.

Nasertorabi, F., Alonso, A., Rogers, S.W., Mustelin, T., Vuori, K., Liljas, L. & Ely, K. R. (2005) Crystallization of the SH2-binding site of p130Cas in complex with Lck, a Src-family kinase. **Acta Cryst**. F61:174-177.

Briknarova, K., Nasertorabi, F., Havert, M. L., Eggleston, E., Hoyt, D. W., Vuori, K. & Ely, K. R. (2005) The serine-rich domain from p130Cas is a four-helix bundle. **J. Biol. Chem.** 280:21908-21914.

Nasertorabi, F., Tars, K., Becherer, K., Kodandapani, R., Liljas, L., Vuori, K. & Ely, K. R. (2006) Molecular basis for regulation of Src by the adaptor protein p130Cas. **J. Mol. Recognit.** 19:30-38.

Derunes, C., Burgess, R., Iraheta, E., Kellerer, R., Becherer, K., Gessner, C. R., Li, S., Hewitt, K., Vuori, K., Pasquale, E. B., Woods, V. L. & Ely, K. R. (2006) Molecular determinants for interaction of SHEP1 with Cas localize to a highly solvent-protected region in the complex. **FEBS Lett.** 580:175-178.

Task 2 (addressing Aim 1).

- Alternative strategy if studies in Task 1 yield negative results: Utilize a novel mammalian screening method to clone proteins that directly interact with Cas and mediate antiestrogen resistance (months 3-36).
  a. Generate MCF-7 cells expressing CasCT-ZIP, CasSD-ZIP and the "leucine zipper" library, and isolate library clones that promote cell proliferation in the presence of tamoxifen.
  b. Reshuttle isolated clones back to CasCT-ZIP-, CasSD-ZIP- and control-transfected MCF-7 cells to confirm the specificity and Cas-dependency for tamoxifen resistance induced by the library clone.
  c. Identify and characterize full length cDNAs for the library clones, and identify the functional role of the cloned protein products in antiestrogen resistance.

Outcome of studies outlined in Task 2:

Tasks 1 and 2 were proposed in the original application as either/or basis. Thus, should results from Task 1 studies have been negative, we would have initiated studies outlined in Task 2. As noted above, studies outlined in Task 1 have been successfully completed. As such, there was no need initiate or perform studies outlined in Task 2 in the context of this application. This notion has been made and approved in the annual reports for years 1 and 2.

Task 3 (addressing Aim 2).

Identify tyrosine residues in Cas that become phosphorylated in breast cancer cells (months 3-36).
a. Express and affinity-purify GST- and myc-tagged forms of truncated (SD-Ser-SBS) and full-length Cas constructs in MCF-7 cells.
b. Prepare tryptic peptides from the immunoprecipitated and SDS gel-separated Cas by in-gel digestion.
(c. Alternatively, utilize immobilized trypsin digestion on the complex.)
d. Carry out IMAC chromatography on the digests and elute phosphopeptides with ammonium hydroxide.
e. Carry out MALDI-TOF mass spectrometry on the bound and unbound fractions.
f. Compare the experimentally determined masses of the peptides to the theoretical masses of the tryptic peptides derived from the Cas sequence to identify phosphorylated peptides and sites.
g. Carry out MS/MS peptide sequencing with ESI-3Q on the peptides assigned by MALDI-TOF MS as containing phosphorylation sites.
h. Carry out precursor ion scanning of the Cas peptides to further confirm the phosphorylation site assignments.

Outcome of studies outlined in Task 3:

We have completed the originally proposed studies outlined in Task 3, and are in progress with continuation studies beyond what was proposed in the DoD application for a (quality) manuscript completion.

Mass spectrometry has evolved as a superior method for phosphorylation site mapping, as the sensitivity of mass spectrometry for peptide sequencing is at least 10 to 100 times more sensitive than traditional Edman degradation methodologies. We have employed two types of mass spectrometers in a multi-tiered strategy to systematically map the *in vivo* phosphorylation sites of Cas in ER-positive MCF-7 breast cancer cells. These include matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) and electrospray ionization triple quadrupole (ESI-3Q) instruments.

An important aspect of this work was our ability to enrich phosphorylated Cas from cell lysates. Immunoprecipitation was used for the initial enrichment from MCF-7 cell lines overexpressing full-length Cas or a truncated version encompassing the SD and SBS domains of Cas. This form of Cas encompasses residues 119-705 and contains only the SD-region, the intervening serine-rich region and the SBS-domain (Src-binding domain). Our previous studies had indicated that the main tyrosine phosphorylation sites in Cas are in the SD-region, and the SBS-domain is required for Src binding and tyrosine phosphorylation of the SD region. Starting with a smaller construct containing the predicted regions of Cas that are phosphorylated provided the advantage that the tryptic peptide mixtures prepared for phosphopeptide mapping were less complex. This translated to a lower possibility of peptides having identical or similar mass values. This in turn was important since our initial efforts were to use comparative mass mapping as a screen for phosphopeptides by comparison with theoretical peptide masses derived from the Cas sequence after enrichment on phosphopeptide trapping columns. After identification of phosphorylation sites in SD-Ser-SBS construct, these sites were then confirmed in full length Cas.

Phosphopeptides bind to immobilized metal affinity chromatography (IMAC) columns with selectivity depending on the transition metal ion used. It has been demonstrated that the most selective transition

metal ion for this purpose is Gallium. Tryptic peptides were prepared from the immunoprecipitated and SDS gel-separated Cas-SD-SBS by in-gel digestion. Next, tryptic peptides were passed over Poros Ga (III) IMAC columns. Phosphopeptides bound to the column were eluted with 0.075% ammonium hydroxide, and the bound and unbound fractions were analyzed by MALDI-TOF mass spectrometry. The experimentally determined masses of the peptides were subsequently compared to the theoretical masses of the tryptic peptides derived from the Cas sequence; mass shifts of 80 Da are indicative of a phosphorylated peptide. A simple method then exists to distinguish tyrosine vs. serine or threonine phosphorylated peptides by comparison of spectra collected in linear and reflector mode. Reflector spectra exhibit the metastable decomposition of 80 Da [MH-HPO_4] for phosphotyrosine and 98 Da [MH-H_3PO_4] for serine and threonine phosphorylated peptides from the parent peptide mass [MH]. Linear spectra do not exhibit this effect showing only the parent ion [MH] mass.

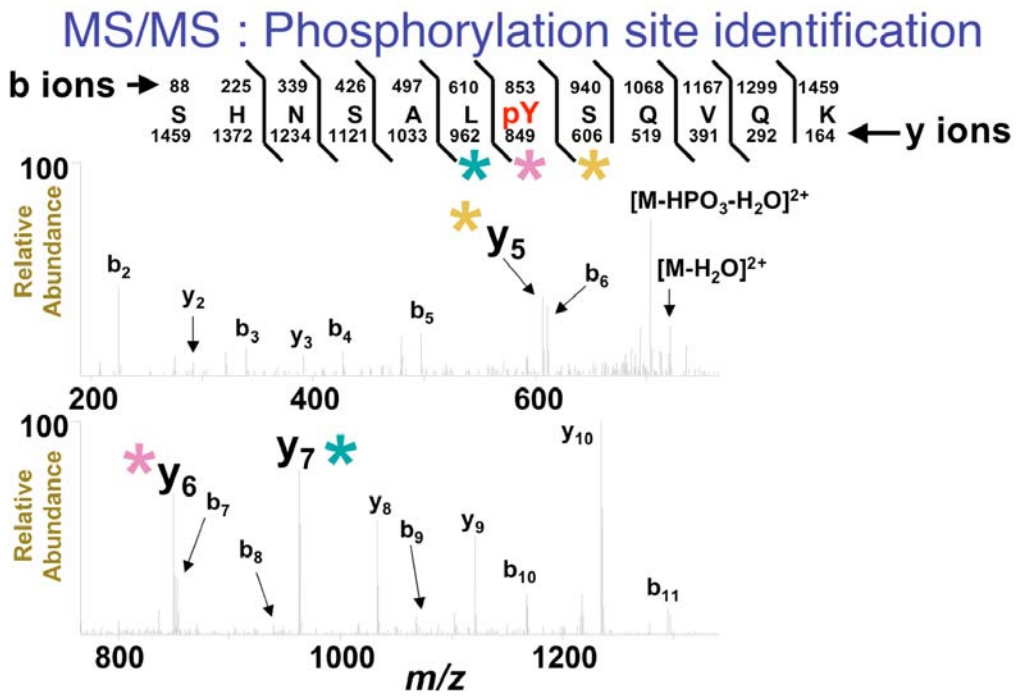


Figure 5. Example of phosphorylation site identification. See text for details.

All tentative phosphorylation site assignments were confirmed by more rigorous methods using ESI-3Q mass spectrometry. As a first step, all peptides tentatively assigned by MALDI-TOF MS as containing potential phosphorylation sites were confirmed by MS/MS peptide sequencing (see Fig. 5). In addition to sequencing of peptides, triple quadrupole mass spectrometers have the ability to perform precursor ion scanning. This scan mode allows the detection of unique modifications such as phosphorylation and glycosylation in peptides. Fragmentation of phosphate (79 Da negative ion mode) from a peptide in the second quadrupole can be monitored in the third quadrupole while scanning a mixture of peptides in the first quadrupole. Thus, Ga(III) column eluates were analyzed on the nanoES ionization source using this orthogonal scan mode after reversed phase micropurification. These experiments confirmed our results from MALDI-TOF analysis.

By applying the methods described above, we have mapped the following tyrosine residues to be phosphorylated in Cas in MCF-7 cells: 238, 253, 271, 291, 310, 331, 391, 414. Importantly, these sites correspond to the sites that match strong recognition consensuses for Sh2 domains of the downstream

effectors of the Crk and Nck families. Importantly, we confirmed that these sites are functional for breast cancer cell signaling. Studies performed by our Proteomics Facility together with GNF indicate that the same sites in Cas become phosphorylated upon IGF-1 stimulation of MCF-7 cells, as assessed by global phosphoproteomics analysis (Fig. 6 below). (Note that numbering is "off" by three amino acids, depending on whether the database sequence contained the ATG codon for the initiation methionine or not).

Figure 6. Global phosphoproteomics analysis of MCF-7 cells stimulated with IGF-1.

PEPTIDE SEQUENCE	PHOSPHO. SITE(S)	PROTEIN	NCBI gi number	IGF1 stimul.	TBS control stimulation
K.VADFGLS*R.L	S385	ABL1 (Proto-oncogen	125135	1 (of 8)	0 (of 4)
K.Y*ATPQVIQAPGPR.A	Y234	Ack1 (similar to activ	8922075	3	2
R.T*PSPLVPPGSSPLPPR.L	T790	Ack1 (similar to activ	8922075	0	0
K.VSSTHYY*LLPERPSYLER.Y	Y858	Ack1 (similar to activ	8922075	0	0
R.AVENQY*SFY.-	Y629	adaptor protein with	10280626	2	0
R.VY*IHPF.-	Y2	ANG2_BOTJA ANGIO	1703308	4!	0!
R.HGS*GADS*DYENTQSGDPLLGLEGK.R	S11S15	ARF GAP GIT1 [Hom	4691726	0	0
R.HGS*GADS*DY*ENTQSGDPLLGLEGK.R	S11S15Y17	ARF GAP GIT1 [Hom	4691726	0	2
R.HGSGADS*DY*ENTQSGDPLLGLEGK.R	S15Y17	ARF GAP GIT1 [Hom	4691726	1	0
K.SLSS*PTDNLELSLR.S	S362	ARF GAP GIT1 [Hom	4691726	2	2
R.QPPGPVPT*PPLPSER.A	T480	ARF GAP GIT1 [Hom	4691726	0	0
R.ILT*MRSELR.A	T333	aspartate aminotrans	4504067	0	1
K.AQQGLY*QVPGPSPQFQSPPAK.Q	Y128	BCA1; p130CAS [Hor	8134341	0	0
R.VGQGYVY*EAAQPEQDEY*DIPR.H	Y224Y234	BCA1; p130CAS [Hor	8134341	0	1
R.VGQGYVYEAAQPEQDEY*DIPR.H	Y234	BCA1; p130CAS [Hor	8134341	2	2
R.HLLAPGPQDIY*DVPPVR.G	Y249	BCA1; p130CAS [Hor	8134341	2	2
K.GPNGRDPLLEVY*DVPPSVEK.G	Y287	BCA1; p130CAS [Hor	8134341	0	0
R.EETY*DVPPAFAK.A	Y327	BCA1; p130CAS [Hor	8134341	0	0
R.RPGPGTLY*DVPR.E	Y387	BCA1; p130CAS [Hor	8134341	1	0
R.VLPPEVADGGVVDSGVY*AVPPPAER.E	Y410	BCA1; p130CAS [Hor	8134341	1	1
R.(SS)*TPLHS*PSPIR.V	S283 or 284, S289	BIS (Bcl-2-binding pr	6724086	0	1
R.(TGS)*EPALSPAVVR.R	T368 or S370	breast cancer antiest	37183110	0	0
R.(TGS)*EPALS*PAVVR.R	T368 or S370S375	breast cancer antiest	37183110	1	1
R.TGSEPALS*PAVVR.R	S375	breast cancer antiest	37183110	0!	2!
K.QPES*MNVTR.N	S205	c-mer proto-oncogen	5453738	0	0
K.GT*FT*LHVPQDTER.L	T525T527	cartilage intermediat	4502845	0	1
K.LY*QVPNPQAAPR.D	Y92	CasL; HEF 1; neural	8134360	7	3
R.TGHGY*VYEYPSR.Y	Y164	CasL; HEF 1; neural	8134360	0	0
R.TGHGYVY*EYPSR.Y	Y166	CasL; HEF 1; neural	8134360	2	1
K.DVY*DIPPSHTTQGVY*DIPPSSAK.G	Y177Y189	CasL; HEF 1; neural	8134360	0!	4!
R.YQKDVY*DIPPSHTTQGVY*DIPPSSAK.G	Y177Y189	CasL; HEF 1; neural	8134360	0!	1!
K.GPVFSVPVGEIKPQGVY*DIPPTK.G	Y214	CasL; HEF 1; neural	8134360	0	0
R.DGVY*DVPLHNPPDAK.G	Y345	CasL; HEF 1; neural	8134360	4	1
R.DLVDGINRLS*FSSTGSTR.S	S369	CasL; HEF 1; neural	8134360	2	1
R.GGS*PLAAPQGGS*PTK.L	S267S276	catenin (cadherin-as	11034811	2	3
R.GGS*PLAAPQGGS*PTKLQR.G	S267S276	catenin delta 2 [Hom	11034811	0	0
R.TSTAPSS*PGVDSVPLQR.T	S461	catenin delta 2 [Hom	11034811	0	0
R.ASYAAGPASNY*ADPYR.Q	Y499	catenin delta 2 [Hom	11034811	7	4
K.DY*ETYQPFQNSTR.N	Y1154	catenin delta 2 [Hom	11034811	2	1
K.STGNY*VDFYSAAR.P	Y1197	catenin delta 2 [Hom	11034811	5	3
K.IGEG*TY*GVVYK.A	T14Y15	CDK2; Cdc2 (cyclin-d	16936530	0	1
K.IGEGTY*GVVYK.A	Y15	CDK2; Cdc2 (cyclin-d	16936530	1	0
K.TQT*PPVSPAPQPTEER.L	T401	Cortactin (Src substr	2498954	0	1
K.TQT*PPVS*PAPQPTEER.L	T401S405	Cortactin	2498954	4	3
R.AKTQT*PPVS*PAPQPTEER.L	T401S405	Cortactin	2498954	0	0
R.LPSS*PVY*EDAASFK.A	S418Y421	Cortactin	2498954	0	2
R.LPSSPVY*EDAASFK.A	Y421	Cortactin	2498954	2	2
R.NLYAGDY*YR.V	Y802	discoidin receptor tyr	7669485	0	0
K.LDQPVS*APPS*PR.D	S218S222	DNA segment on chr	22477628	0!	3!
K.VVEAVNS*DS*DSEFGIPK.K	S613S615	DNA topoisomerase (	4218208	2	4
K.VVEAVNS*DSDS*EFGIPK.K	S613S617	DNA topoisomerase (	4218208	0	0

Following completion of Task 3 and identification of the phosphorylation sites in Cas as proposed in the application, several lines of additional investigation will be undertaken. First, we will generate antibodies that are specific for given phosphorylation sites in Cas. Based on our preliminary data (see above), we anticipate that these reagents will provide enhanced specificity and sensitivity in prognostic evaluation of antiestrogen resistance in breast cancer. We have significant experience in antibody production and validation, and these techniques are not described here. We also have access to clinical material for a thorough evaluation of the use of the antibodies, alone or in combination, in assessing Cas phosphorylation status in breast cancer cells and in prognostic analysis for antiestrogen therapy.

In sum, accomplishment of Tasks 1 and 3 during the DoD grant funding period provides a solid foundation for exciting future studies, extending the scope of the original proposed project.

3. Key Research Accomplishments

1. Generation of all of the DNA constructs needed to accomplish studies outlined in the original application.

2. Examination and confirmation of the working model of this grant application; that is, that the Rac pathway forms a common pathway downstream of the Cas/Crk/BCAR3 signaling complex to mediate antiestrogen resistance in breast cancer. (Manuscript in preparation upon completion of additional studies beyond the DoD grant that are outlined above).

3. Discovery of a novel role for Cas in regulating the G1-S transition in mammalian cells.

4. Mass spectrometry-based mapping of tyrosine phosphorylation sites of Cas in breast cancer cells (manuscript in preparation upon completion of additional studies beyond the DoD grant that are outlined above).

4. Reportable Outcomes

1. Generation of molecularly-defined key DNA constructs and breast cancer cell lines to examine antiestrogen resistance *in vitro*.

2. Generation of molecularly-defined cell lines that are genetically deficient of Cas.

3. Publication: see Appendix.

5. Conclusions

Our data presented in the original grant application supported the role of the docking protein Cas in antiestrogen resistance. During the period of this grant funding, we have confirmed the role for Cas in antiestrogen resistance in cell lines, and also found that BCAR3 and Crk, via Rac, are essential components of mediating the effects of Cas in this pathway. These studies complete Task 1. Additionally, we have expanded our goals to have cell cycle studies as they relate to G1-S transition as an additional read-out. Task 2 was on either/or basis with Task 1, and was not addressed. Task 3, mapping of phosphorylation sites in Cas, has also been completed. Together, these findings provide a solid base for future work by us and others, beyond the scope of the original application.

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7. Key Personnel

Current period (Yr 3)

Kristiina Vuori, PI	6%
Darren Finlay, Postdoc	45%
Ericka Eggleston, Lab Mgr	50%
Kirsi Perrine, Graduate Student	50%
Sahand Mirzahossein, Research Technician	100%

Year 2:

Kristiina Vuori, PI	7.5%
Kirsi Perrine, Graduate Student	50%
Sahand Mirzahossein, Research Technician	100%

Year 1:

Kristiina Vuori, PI	10%
Ericka Eggleston, Lab Mgr	25%

Kirsi Perrine, Graduate Student	100%
Sahand Mirzahossein, Research Technician	75%

8. Appendix

Dail, M., Kalo, M. S., Seddon, J. A., Côté, J. F., Vuori, K. & Pasquale, E. B. (2004) SHEP1 function in cell migration is impaired by a single amino acid mutation that disrupts association with the scaffolding protein Cas but not with Ras GTPases. **J. Biol. Chem.** 279:41892-41902.